Human Vaccines & Immunotherapeutics 9:11, 2378–2385; November 2013; © 2013 Landes Bioscience

Formulation and immunological evaluation of a trivalent vaccine comprising emulsified submicron particles and inactivated virions of H5N1/EV71/JEV

Chih-Wei Lin[1], Ching-Yun Chang[1], Wei-Lin Chen[1], Shih-Chang Lin[2], Chien-Chun Liao[1], Jui-Yuan Chang[1], Chia-Chyi Liu[1], Alan Yung-Chih Hu[1], Tsung-Chun Lu[1], Ai-Hsiang Chou[1], Suh-Chin Wu[2], Pele Chong[1,2,3,*], and Ming-Hsi Huang[1,*]

[1]National Institute of Infectious Diseases and Vaccinology; National Health Research Institutes; Zhunan, Taiwan; [2]Institute of Biotechnology; National Tsing Hua University; Hsinchu, Taiwan; [3]Graduate Institute of Immunology; China Medical University; Taichung, Taiwan

Keywords: adjuvant, combination vaccine, enterovirus 71, influenza, Japanese encephalitis

Combination vaccines can reduce the number of injections and simplify the immunization schedule required to prevent different diseases. Here we assessed the immunogenicity in a mouse model of a vaccine composition comprising inactivated influenza viruses (H5N1/H1N1), enterovirus 71 (EV71), and/or Japanese encephalitis virus (JEV) and investigated whether the vaccine formulations can overcome the immunologic interference between the individual vaccine components. We demonstrated that the antigenic competition happens between H5N1/H1N1 or H5N1/EV71 inactivated virions when the vaccine combinations either formulated with Alum suspensions or without adjuvant. In the presence of PELC emulsified particles, EV71-specific immune responses before and after incorporating H5N1 virus into EV71 vaccine were detected of no significant difference; in addition, H5N1- and EV71-specific immune responses were found at the same level when H5N1/EV71/JEV consolidating into combination vaccine. Emulsified vaccine formulation was represented as a potential tool that is found to reduce the number of injections required to prevent multiple infectious strains causing the same disease (H5N1/H1N1) and/or that protect against different diseases (H5N1/EV71). Combination vaccines can also include a third component to protect against H5N1/EV71/JEV at the same time.

Introduction

Vaccination is the best cost-effective biomedical approach against infectious diseases. Typically, a vaccine contains an agent dubbed antigen that resembles a disease-causing microorganism (or pathogen) against which adaptive immune responses are elicited. To cover a wide range of diseases with the minimum number of parental injections, the simple way is to make a combination or multivalent vaccine by mixing the different vaccine antigens and injecting them simultaneously.[1,2]

Presently, a number of combination vaccine products included in the immunization schedules for children.[3,4] For example, diphtheria toxoid-tetanus toxoids-acellular pertussis vaccine (Tripedia® and Daptacel® by Sanofi-Pasteur; Infanrix® by GSK) and measles-mumps-rubella vaccine (M-M-R® II, Merck). Recent years, combination vaccines include increasing numbers of components to protect against these and other diseases, including inactivated polio vaccine (Kinrix®, GSK), *hemophilus influenzae* type b (Pentacel®, Sanofi-Pasteur), hepatitis B (Pediarix®, GSK), and varicella (ProQuad®, Merck). Combination vaccines are also designed to immunize against two or more strains of the same pathogens, such as trivalent inactivated influenza vaccine (TIV) and pneumococcal polyvalent conjugate vaccine (Streptococcus pneumoniae serotypes, Prevnar™, Wyeth).

In order to implement the policy of the government for developing vaccine self-manufacturing capability and responding to any pandemic outbreak, and also for strengthening the infrastructure of local important vaccine research and production, the NHRI Vaccine Center is currently developing cell culture based platform technology to quickly produce vaccines against emerging infectious diseases such as MDCK cell-based H5N1/H1N1/H7N9 influenza vaccine, and Vero cell-based enterovirus (EV) 71 and Coxsackie virus A16. For the reason that currently licensed JEV vaccines were in a predicament in terms of manufacturing process, we use the same cell culture technology to produce Vero cell-based Japanese encephalitis virus (JEV) vaccine. It is hoped that these vaccine candidates could be included in the vaccination programs in the future when the vaccines become available. As an increase number of new and improved vaccines are being introduced to prevent childhood diseases, the current trend is to develop combination vaccine including other components in different arrays to prevent different diseases and/or that protect against multiple strains of infectious agents causing the same disease.[4] Since JEV and

* Correspondence to: Pele Chong, Email: pelechong@nhri.org.tw; Ming-Hsi Huang, Email: huangminghsi@nhri.org.tw
Submitted: 05/16/2013; Revised: 06/19/2013; Accepted: 07/05/2013
http://dx.doi.org/10.4161/hv.25639

EV71 are the leading causes of acute viral encephalitis in the Asia-Pacific region, a bivalent or multivalent vaccine that can provide protection against JEV and prevalent enteroviruses are desirable. Moreover, it may logically be co-administered with other components in different arrays to defense certain vaccine-preventable diseases, e.g., influenza. However, the development of combination vaccines is not straightforward. Different antigens are merged into one vaccine formulation could be difficult to overcome chemical incompatibility and immunologic interference.[4,5] Regarding this, incorporation of vaccine adjuvants (Latin adjuvare, "to help") to vaccine candidates is a potential strategy to bypass these limitations.[6]

Vaccine adjuvants are conferred on the substance that helps the host to elicit a robust and effective antigen-specific immune response. They are added to many vaccines to increase their immunogenicity and efficacy.[7] Conventional aluminum-based mineral salts (Alum) and water-in-oil (W/O) emulsions however have the limitations in terms of adjuvanticity and safety, confronting emergency/massive vaccination.[8] Regarding this, the World Health Organization (WHO) had highlighted that oil-in-water (O/W) emulsions are very effective adjuvants for pandemic influenza vaccine preparedness.[9] In order to deliver effectively the vaccine antigens and/or immunostimulatory molecules to the immune cells, we optimized an emulsified vaccine formulation comprised of a bioresorbable polymer poly(ethylene glycol)-*block*-poly(lactide-co-ε-caprolactone) (PEG-*b*-PLACL), Span®85, and squalene to form a ready-to-use adjuvant, dubbed PELC.[10] The immunogenicity studies assessed in mice using an inactivated H5N1 viral antigen have demonstrated that PELC allowed for antigen dose sparing and increased seroconversion and cross-protection.[10,11] The use of PELC could be an effective tool for pandemic vaccine preparedness and could thus play an important role in single-dose immunization. However, whether emulsified vaccine formulation can overcome the interference between individual antigens is still unknown.

This study relates to a combination vaccine against three virus infectious diseases, including influenza, enterovirus diseases, and Japanese encephalitis. The antigens used here are the formalin-inactivated whole viruses, which were propagated in the serum-free cell culture-based technology. The vaccine compositions comprise an inactivated avian A(H5N1) influenza viral antigen and optionally in addition one of the following: first, an inactivated 2009 pandemic influenza A(H1N1) influenza viral antigen; second, an inactivated enteroviral antigen; and third, a combination of an inactivated enteroviral virion and an inactivated JEV virion. For the feasibility study on a combination vaccination, it will be important to evaluate whether the use of adjuvants can prevent the immunologic interference between the antigens. The vaccine candidates are further formulated with PELC emulsified particles or adjuvant combination consisted of PELC and an immunostimulatory agent, CpG (oligodeoxynucleotide containing unmethylated cytosine-guanosine motifs).[12] The immunogenicity of a combination vaccine candidate after formulated with PELC and/or CpG was determined in mice for induction of humoral and cellular responses following prime/boost or single-dose immunization schedules. The results were compared with those obtained without adjuvant or with conventional Alum suspensions.

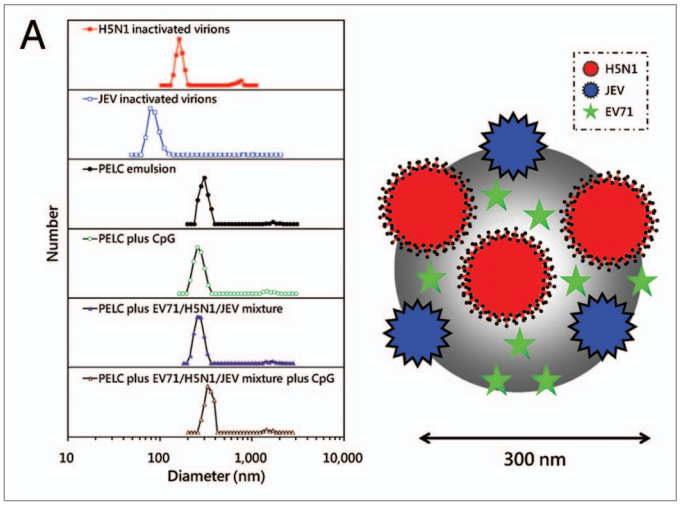

Figure 1. Laser light scattering analysis and schematic representation of the vaccine compositions and PELC-emulsified vaccines. Formalin-inactivated H5N1 virus suspensions have the size of 150 nm with some aggregates. JEV virion is provided with the diameter below 100 nm. EV71 virion possesses the size below the detection limit. On the other hand, homogeneous fine particles with mean size ranged from 200 to 400 nm were observed in the PELC-emulsified vaccines. Data are representative for at least five independent experiments.

Results

Formulating inactivated virions with PELC emulsion

The antigens used here are the cell-based formalin-inactivated whole virions, which were produced in a well-optimized vaccine manufacturing bioprocess involving upstream virus growth and downstream purification (see **Materials and Methods: Vaccine preparation**). The size distribution of the vaccine compositions was investigated by laser light scattering. As shown in **Figure 1**, formalin-inactivated H5N1 virus suspensions were composed of rather nonhomogeneous particles with a bimodal distribution. Two different sizes were observed, the relatively large particles of 1000 nm and smaller ones of 150 nm. This finding was assigned to the fact that some viral aggregates were associated during concentration/purification. The dynamic light scattering pattern of JEV virions showed a unimodal distribution with a diameter of 80 nm, in agreement with literature. We have previously determined the EV71 viral particle size being 30–35 nm in diameter by transmission electron microscopy;[13] however, EV71 virion possesses the size below the light scattering detection limit.

After formulated with PELC emulsion, the vaccine compositions were observed homogeneous submicron particles with mean diameter ranged from 200 to 400 nm (**Fig. 1**). Virus antigens and/or CpG are not a key point to the particle size of the emulsions, probably due to small amount of antigen or CpG content in the vaccine formulations. Importantly, some authors reported that this scale of dimension is favorable to uptake by antigen-presenting cells (APCs), facilitating induction of potent

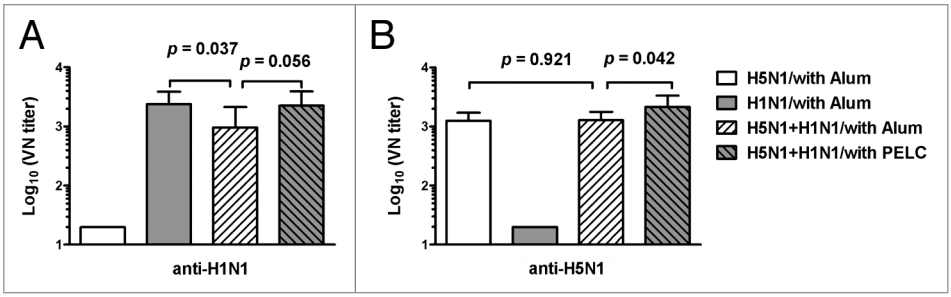

Figure 2. (**A**) H1N1-specific, (**B**) H5N1-specific virus neutralizing antibodies elicited in mice following immunization with inactivated virus vaccine. BALB/c mice were vaccinated i.m. twice at day 0 and day 14 with the candidate vaccine formulations. At day 21, sera were collected from blood and incubated with a H5N1 or a H1N1 virus strain. Data are presented as mean titers with standard deviations of six mice per group; an undetectable level was scored as a titer equal to 20; the p values were calculated using a two-tailed Student's t-test.

immune responses.[6,14] It is also worth noting that the particle size of Alum suspensions was not detectable by using light scattering technology due to high heterogeneity.

H5N1/H1N1 bivalent vaccine

First of all, the immunogenicity studies were conducted in mice to prevent multiple strains of infectious agents causing the same disease. We use inactivated H5N1 and H1N1 viruses as model antigens. As shown in **Figure 2**, the mice that received two doses of monovalent H5N1 inactivated virus vaccine could not generate cross-neutralizing antibodies against H1N1; in the meantime, the mice that received H1N1 virus could not generate cross-neutralizing antibodies against H5N1. These findings demonstrated that the H1N1 virion was antigenically distinguishable from the H5N1 virion, a bivalent combination vaccine is required to protect against H5N1/H1N1 virus infections. However, the anti-H1H1 specific immune responses were rather reduced when incorporating an inactivated H5N1 virus into inactivated H1N1 virus vaccine using Alum adjuvant (**Fig. 2A**); meanwhile, the incorporation of an inactivated H1N1 virus into inactivated H5N1 virus vaccine did not influence the anti-H5H1-specific immune responses (**Fig. 2B**). Interestingly, mixing the same antigens with PELC can generate the same level of neutralizing antibodies against both influenza A viruses, indicating PELC has potential of overcoming immunologic interference of H5N1/H1N1.

H5N1/EV71 bivalent vaccine

To evaluate the potential of overcoming antigenic interference, we next examine the effects of the incorporation of an inactivated H5N1 virus into EV71 vaccine on the immunogenicity of the resulting combination vaccines. As expected, inactivated EV71 virus alone (no H5N1 antigen) did not generate H5N1-specific antibody immune responses (**Fig. 3A and B**), indicating the EV71 virion was antigenically distinguishable from the H5N1 virion. The anti-H5H1 specific immune responses were generated when incorporating the inactivated H5N1 virus into inactivated EV71 virus vaccine. However, the seroprotection of the mice immunized with inactivated virus alone did not reach 100% until 8th week (**Fig. 3A**). On the other hand, PELC can enhance significantly the protecting H5N1 immune responses compared with inactivated virus alone ($p < 0.05$). Moreover, the H5N1 seroprotection rate easily reached 100% at Week 4, 8, 12 for the mice received PELC-adjuvanted H5N1/EV71 vaccine. **Figure 3B** shows the EV71 antibody responses in BALB/c mice immunized with inactivated H5N1/EV71 viruses. Following a single injection, sera from mice vaccinated with 0.2 μg of EV71 inactivated virus alone cannot elicit effective responses. The incorporation of an inactivated H5N1 virus into inactivated EV71 virus vaccine did not influence the EV71-specific immune responses. In the case of PELC-formulated vaccines, the antibody titers were dramatically enhanced when the mice immunized with the same amount of antigens. The anti-EV71 immune responses were rather reduced at Week 4 when incorporating an inactivated H5N1 virus. Moreover, no significant difference was detected between the groups before and after incorporating H5N1 virus into EV71 vaccine, indicating that PELC has potential of overcoming antigenic interference between H5N1/EV71.

Another advantage of vaccination with PELC-formulated vaccines was also revealed after incorporating CpG. As shown in **Figure 4A**, significant difference in protective anti-H5N1-immune response was found in the groups of mice that received PELC/CpG- or Alum/CpG-formulated H5N1/EV71 vaccine with respect to the group formulated with CpG. However, significant enhancement of anti-EV71-immune response was found only in the group of PELC/CpG-formulated vaccine with respect to the group formulated with CpG (**Fig. 4B**); the features of adjuvanticity of Alum/CpG for anti-EV71 responses are less pronounced than for anti-H5N1 responses.

H5N1/EV71/JEV trivalent vaccine

To study the immunogenicity of the combination vaccines, we further extend our examination of the effects on the incorporation of a third inactivated virus, JEV, into H5N1/EV71 as a trivalent vaccine. The immunogenicity study of an inactivated JEV whole-virion vaccine formulated with emulsified particles was performed in mouse. The elicited antigen-specific antibodies are shown in **Figure 5**. Following a single injection, 0.5 μg of non-adjuvanted JEV vaccine elicited low IgG titers against JEV (GMT less than 1000) within 4 weeks. The anti-JEV IgG titer increased slightly to 1200 at Week 8 and then decreased to 900 at Week 12. When the same amount of inactivated JEV vaccine candidate was formulated with PELC, the induced anti-JEV IgG titers were significantly higher than those induced by non-adjuvanted inactivated virus ($p < 0.05$). The elicited antigen-specific IgG titer was 12000 at Week 4, then to 36000 (30 times more than non-adjuvant group) at Week 8, and then to 42000 (45 times more than non-adjuvant group) at Week 12, respectively. To the best of our knowledge, immunity elicited by cell-based emulsion-formulated JEV vaccine has not reported in the literature. Here we found that the number of JE vaccination could be minimized by

way of enhancing the antigen-specific immune responses via adjuvant strategy. We propose this vaccine can ready be given at the same time with other adjuvanted vaccine.

Figure 6 shows the effects of the incorporation of an inactivated JEV virus on the immunogenicity of the H5N1/EV71 combination vaccines. We found that the anti-EV71 specific antibody responses were significantly enhanced after the incorporation of JEV into H5N1/EV71 bivalent vaccine in the case of non-adjuvanted vaccines ($p < 0.05$), whereas the anti-H5N1 specific antibody responses were slightly decreased (**Fig. 6A**). For PELC-adjuvanted vaccines, the elicited EV71- and H5N1-specific antibody responses were significant higher than those observed by non-adjuvanted vaccines; furthermore, both anti-H5N1 and anti-EV71 antibody titers were in the same level after the incorporation of JEV, a feature of overcoming antigenic competition. In so far as the rationale of H5N1/EV71/JEV trivalent vaccine was concerned, the mice simultaneously generate the immune responses against H5N1, EV71 and JEV after receiving a single injection of H5N1/EV71/JEV trivalent vaccine (**Fig. 6B**). Moreover, we found that the trivalent vaccine candidate required vaccine adjuvant to elicit high titers of H5N1- and JEV-specific serum antibodies, PELC and Alum as well.

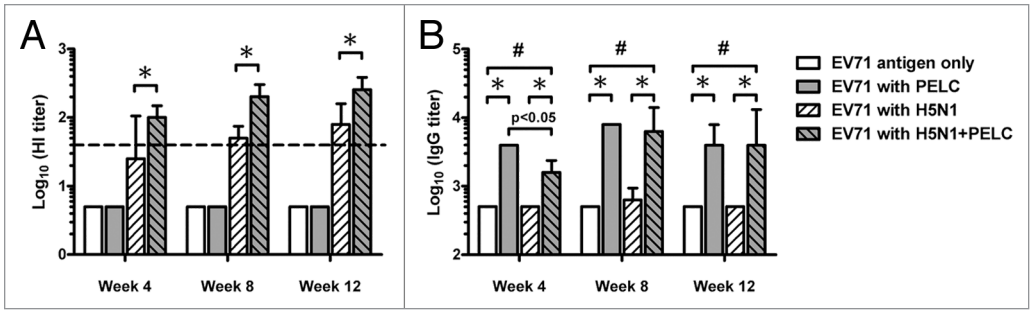

Figure 3. (**A**) H5N1-specific HI, (**B**) EV71-specific IgG antibodies elicited in mice following immunization with inactivated virus vaccine. BALB/c mice were vaccinated i.m. once with the candidate vaccine formulations. Serum samples from a pool of two mice were collected from immunized mice and the antibody titers were determined by immunoassays. Data are presented as mean titers with standard deviations of six mice per group; the dotted horizontal line represents an HI titer of 40. Statistical significance was determined by two-way ANOVA Bonferroni post test. *$p < 0.05$: Comparison between the groups before and after formulated with PELC. #$p < 0.05$: Comparison between the groups EV71 antigen only and EV71 with H5N1+PELC.

T-cell responses were measured in the spleen following re-stimulation of cells in vitro with H5N1 antigen. As shown in **Figure 7**, the vaccination of the H5N1/EV71 antigens alone did not induce a notable H5N1-specific response, IFN-γ and IL-4-producing cells were detected slightly higher than the control group. On the other hand, once the PELC-formulated vaccine candidate was administered, a positive cytokine secretion response was induced and the IFN-γ and IL-4-producing cells were much higher than those obtained from the non-adjuvanted group and the Alum-adjuvanted group. Surprisingly, we also found that the incorporation of JEV in the H5N1/EV71 combinations increases the H5N1-specific cytokine secretion responses and allows the host to bias the IFN-γ/IL-4 ratio.

Discussion

Presently, annual vaccination is the most effective intervention to prevent seasonal influenza virus infection and its complications; it is also expected to be effective in controlling pandemic influenza.[15,16] For the first time, we step-by-step investigated the effects of the incorporation of inactivated EV71/JEV whole virions on the immunogenicity of the candidate H5N1 vaccine. Our results revealed that inactivated JE virus may not only act as an antigen to generate the immune response against JEV infection (**Fig. 6B**), but also may play as an adjuvant to enhance the H5N1/EV71-specific humoral immune responses as well as the cytokine

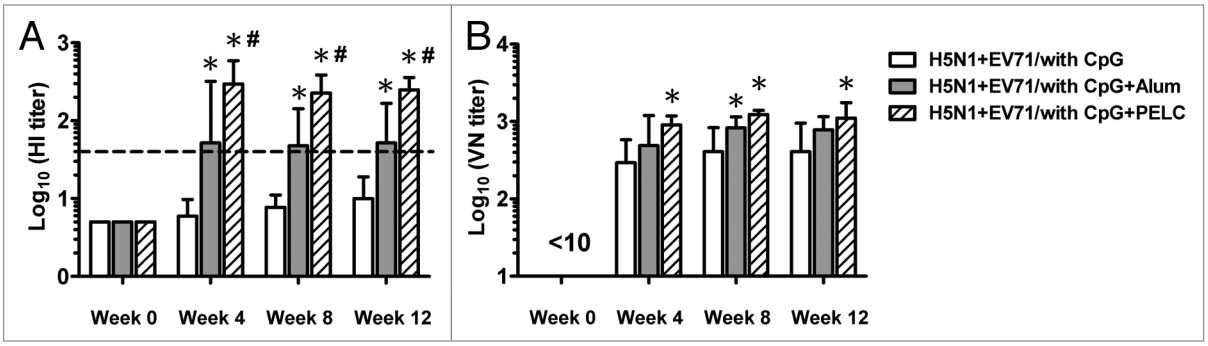

Figure 4. (**A**) H5N1-specific HI, (**B**) EV71-specific VN antibodies elicited in mice following immunization with the inactivated EV71/H5N1 combination in vaccine. BALB/c mice were vaccinated i.m. once with the candidate vaccine formulations. Serum samples were collected from immunized mice and the antibody titers were determined by immunoassays. Data are presented as mean titers with standard deviations of eight mice per group; the dotted horizontal line represents an HI titer of 40. Statistical significance was determined by two-way ANOVA Bonferroni post test. *$p < 0.05$: Comparison with the group of CpG at the same time point. #$p < 0.05$: Comparison with the group of CpG/Alum at the same time point.

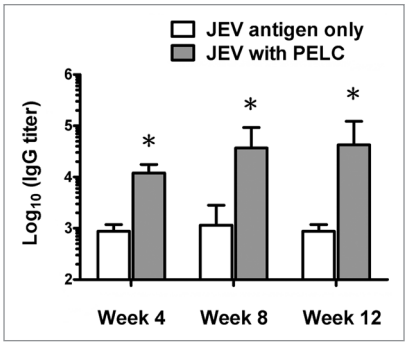

Figure 5. JEV-specific IgG antibodies elicited in mice following a single intramuscular dose of JEV inactivated virus. BALB/c mice were vaccinated i.m. once with dose of 0.5 μg cell-based formalin-inactivated JEV vaccine, with or without PELC emuldsion. Serum samples were collected from immunized mice and the antibody titers were determined by ELISA. Data are presented as mean titers with standard deviations of five mice per group. Statistical significance was determined by one-way ANOVA Bonferroni post test at the same time point. *p < 0.05: Comparison with the group without adjuvant.

secretion responses (**Figs. 6A and** 7). It is commonly believed that protective immunity against influenza virus infection is mediated by neutralizing antibodies, whereas increasing IFN-γ induction via vaccination is an important strategy for limiting the severity of influenza-associated illness by new strains in the absence of specific antibody responses.[15,17] Further study could also be performed to test the possibility to induce cell-based JEV vaccine in the licenses combination vaccines.

Currently, three types of JE vaccines in large-scale use are (A) the inactivated mouse brain-derived vaccine (JE-VAX®, manufactured by Sanofi Pasteur), received as a three-dose regimen; (B) the Alum-adjuvanted, inactivated cell culture-derived vaccine (IXIARO®, manufactured by Intercell

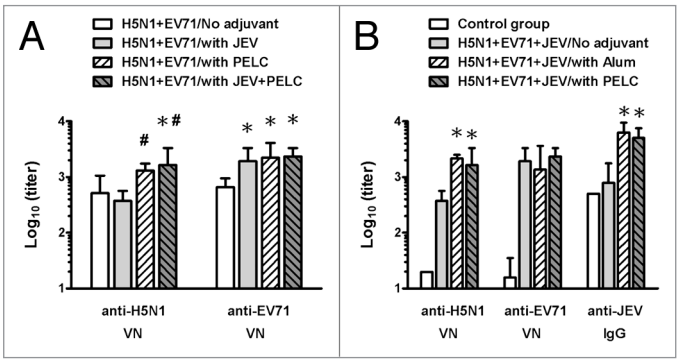

Figure 6. Antigen-specific antibodies elicited in mice following immunization with H5N1/EV71/JEV combination vaccines. (**A**) JEV effect, (**B**) adjuvant effect. BALB/c mice were vaccinated i.m. once with the candidate vaccine formulations. At week 12, serum samples from a pool of two mice were collected from immunized mice and the antibody titers were determined by immunoassays. Data are presented as mean titers with standard deviations of six mice per group. Statistical significance was determined by two-way ANOVA Bonferroni post test. *p < 0.05: Comparison with the group H5N1/EV71 without adjuvant. #p < 0.05: Comparison with the group H5N1/EV71/JEV without adjuvant.

Biomedical), given as a two-dose primary series; and (C) the live-attenuated vaccine based on the SA 14-14-2 strain of the JE virus (IMOJEV®, manufactured by Sanofi-Pasteur), given as a single-dose injection.[18,19] How to minimize the number of vaccine injections by the use of new generation vaccines such as those that are formulated with adjuvants and live-attenuated vaccines is one of the most important strategies to implement H5N1/EV71/JEV vaccination program. Our results manifested the use of PELC emulsified submicron particles were better able than Alum suspensions to enhance the antigen-specific humoral and cellular immune responses (**Figs. 4 and** 7). PELC also has potential to overcome antigenic interference between the individual components of H5N1/EV71/JEV trivalent vaccine, responding the major questions and technical hurdles to a successful combination vaccine (**Figs. 5 and 6**). For designing the new generation vaccines for specific applications, combination adjuvants which consist of particulate delivery system and immunostimulatory compound can be regarded as an interesting alternative to deliver antigen/immunostimulator effectively to the receptors of the immune cells and/or to generate the number of the receptors. It is well-documented in literature that the administration of CpG is agonists of intracellular receptor TLR9 that can induce the activation of antigen presenting cells and Th1-dominated immune response.[12] The present results indicate that it is beneficial of adding CpG to PELC emulsion, instead of Alum suspensions. In fact, it has been shown that small solutes or nanoparticles (< 50 nm) are internalized by APCs through macropinocytosis,[6] whereas poly(lactide-co-glycolide) microparticles (> 500 nm) and MF59 oil-in-water emulsion 200 nm in size can be internalized APCs through phagocytosis without specific recognition has been reported in the literature.[6,14] Particulate vehicles can also be conjugated with alarm/danger signals that bind to pathogen recognition receptors and induce the maturation of APCs.[6]

Beside immunologic interference, the other issues of the rational design of combination vaccine include the presence of preservatives, additives, or manufacturing residuals other than antigens.[1,5] For examples, adverse events have been associated with JEV vaccine due to the use of protamine sulfate during purification, the use of gelatin in freeze-drying, and/or the use of thimerosal in multi-dose containers. To lower the risk of post-vaccination encephalitis as well as hypersensitivity reactions, JEV antigen used in our study was propagated in the cell culture-based technology and purified with Benzonase® endonuclease. Overall speaking, the rationale of the use of emulsified vaccine formulation or adjuvant combination system was to develop an effective single-dose multivalent vaccine. It should be an attractive approach to prevent from introducing other harmful additives such as gelatin stabilizers, antibiotics, or preservatives.

In this study, we substantiated that the emulsified submicron particles can overcome the immunologic interference between the antigens and can enhance the antigen-specific immune responses. In addition, we demonstrated that inactivated JE virions may not only play an antigen to protect the infection of JEV, but also an adjuvant to enhance the H5N1/EV71-specific humoral protection as well as T-cell immunity in mice. Accordingly, these

results will be of great interest for those who work in the field of preventive medicine so as to design the future vaccines against influenza pandemic as well as the tropical infectious diseases, e.g., viral encephalitis associated with JEV and EV71.

Further investigations are required to evaluate whether the use of designed adjuvants can turn on harmful reactions after administration in mice; moreover, it will be important to conduct the immunogenicity as well as virus challenge studies of H5N1/EV71/JEV individual components or trivalent vaccine candidates in transgenic mouse model.

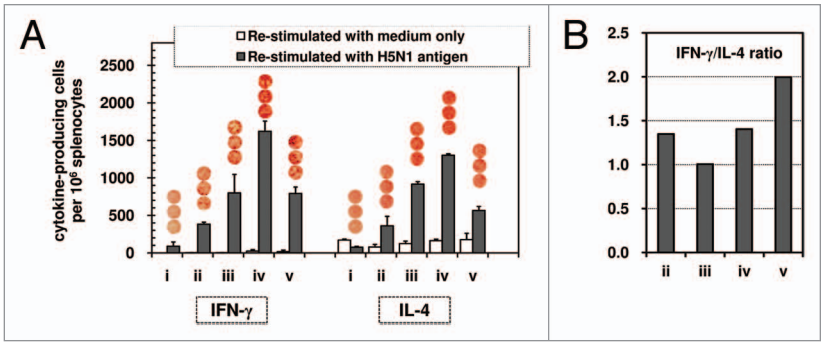

Figure 7. (**A**) Cytokine-producing responses (**B**) IFN-γ/IL-4 ratio in spleen cells following immunization with inactivated H5N1/EV71 viruses without adjuvant or formulated with different adjuvants. (i) control; (ii) no adjuvant; (iii) Alum; (iv) PELC; (v) JEV. BALB/c mice were primed i.m. with a combination vaccine of EV71 inactivated virus 0.2 μg and H5N1 inactivated virus 0.5 μg HA. At day 14, all mice were boosted i.m. with the same vaccine formulations. Seven days after the boost, the splenocyte suspensions were pooled from six mice per group and incubated in the presence or absence of 0.5 μg HA/mL of inactivated H5N1 antigen virus. IFN-γ- and IL-4-producing cells were assessed by enzyme linked immunosorbent spot (ELISpot) assays of cell suspensions for 3 d of culture. Data are expressed as the mean plus the standard deviation of triplicate cultures. The ratio of IFN-γ- and IL-4-producing cells is determined from the difference between the mean spots per well in the presence of antigen and those with medium only.

Material and Methods

Vaccine preparation

The vaccine compositions comprising (1) an inactivated H5N1 influenza virus vaccine, NIBRG-14, derived from a reassorted H5N1 vaccine strain A/Vietnam/1194/2004 and propagated in Madine-Darby canine kidney (MDCK) cells in a microcarrier cell culture; (2) an inactivated H1N1 influenza virus vaccine, NIBRG-121, derived from a reassorted H1N1 vaccine strain A/California/7/2009 and propagated in MDCK cells in a roller bottle cell culture; (3) an inactivated EV71 vaccine derived from a type 71 enterovirus strain E59 (genotype B4) and propagated in African green monkey kidney (Vero) cells in a perfusion bioreactor process; (4) an inactivated JEV vaccine, derived from Beijing-1 strain of JE virus and propagated in Vero cells in a roller bottle cell culture.

Vaccine production flowchart

The antigens used here are the cell-based chemically inactivated whole virions, which were produced in a P2+ manufacturing facility under the well-optimized vaccine manufacturing bioprocess involving cell expansion in serum-free medium, virus inoculation/harvest, downstream purification using sucrose gradient ultracentrifugation, and formalin inactivation.[20-22] The MDCK cells were purchased from Food Industry Research and Development Institute (FIRDI), Taiwan. The Vero cells were purchased from the American Type Culture Collection (ATCC). Master and working cell banks were established following current Good Manufacturing Practices (cGMP) guidelines.

H5N1/H1N1 vaccine preparation

The H5N1 vaccine used was a formalin-inactivated whole virus vaccine, NIBRG-14 (kindly supplied by the UK National Institute of Biological Standard and Control, NIBSC), derived from a reassortant H5N1 vaccine strain A/Vietnam/1194/2004 virus. The viruses were propagated in serum-free media (Cesco) and in the MDCK cell culture-based microcarrier technology. The H1N1 vaccine used was a formalin-inactivated whole virus vaccine, NIBRG-121, derived from a reassorted H1N1 vaccine strain A/California/7/2009. The viruses were propagated in serum-free media (Cesco) and in the MDCK cell culture-based roller bottle technology. Formalin-inactivated vaccines were prepared with 0.1% formalin at 37°C for 24 h. We had performed plaque assay based on plaque forming unit (PFU) in MDCK cells and the $TCID_{50}$ (50% tissue culture infective dose) assay based on the cytopathic effect to evaluate whether there was incomplete formalin inactivation during storage. After the sterilization through a 0.22 μm filter membrane, the HA content of the vaccine bulk was determined by single-radial diffusion (SRD) assay with the standard antigen and antiserum from NIBSC. The antigen medium was prepared with particular HA concentration of the vaccine bulk which is diluted in the phosphate buffered saline (PBS).

EV71 vaccine preparation

The EV71 vaccine used was a formalin-inactivated whole virus vaccine derived from the clinical isolate, E59 strain (genotype B4, kindly given from the Taiwan CDC). EV71/E59 virus stocks were collected from the supernatants of infected Vero cells at three days postinfection. The titers of the master virus seed stocks were determined by plaque assay, and the stocks were stored at -80°C. The production of EV71/E59 virus was done in the serum-free medium (GIBCO) containing 5 g/L of Cytodex 1 based on the microcarrier cell culture bioprocess. Typically, Vero cells were initially inoculated with 2×10^5 cells per mL in a 5-L perfusion bioreactor (NBS), and the cell density reached 2 to 2.5×10^6 cells per mL after six days of cultivation. The Vero cells were infected with EV71/E59 at a MOI of 10^{-5}. EV71/E59 virus was harvested and collected from the microcarrier culture supernatants at either 7 or 8 d post-infection. The cell debris was removed by filtration through a 0.65-μm membrane (Sartorius Stedim Biotech). The crude virus bulk was concentrated 20-fold using a 100-kDa cut-off diafiltration membrane in a tangential flow filter cassette (Sartorius Stedim Biotech), and purified by continuous sucrose gradient ultracentrifugation (Hitachi CP80). The fractions (50 mL per fraction) were pooled, further

concentrated, and then inactivated with 0.025% formalin (v/v) at 37°C for 3 d. The vaccine bulk was obtained after sterile filtration using a 0.22 μm-filter, subjected to SDS-PAGE and western blot analyses, and stored at 4°C. The total protein concentration of the vaccine bulk was determined by the BCA protein assay. Viral titers were determined using the median endpoint of the $TCID_{50}$ by counting the cytopathic effects on infected Vero cells.

JEV vaccine preparation

The JEV vaccine used was a formalin-inactivated whole virus vaccine derived from Beijing-1 strain of JE virus. Vero cells were thawed, and expanded progressively from T25 flask, T75 flask, T150 flask, to 850 cm^2 of roller bottle. The Vero cells were infected with JE virus at a MOI of 10^{-3}. JE virus was harvested and collected at 4 d post-infection. The cell debris was removed by filtration through a 0.65-μm membrane (Sartorius Stedim Biotech). The crude virus bulk was concentrated 20-fold using a 300-kDa cut-off diafiltration membrane in a tangential flow filter cassette (Sartorius Stedim Biotech). The host DNA residues were removed by the treatment with Benzonase® endonuclease. JEV virus concentrate was purified using an ÄKTA Pilot liquid chromatography system (GE Healthcare), and then inactivated with 0.02% formalin (v/v) at 4°C for 30 d. The total protein concentration of the vaccine bulk was determined by Bradford method. Viral titers were determined by a plaque assay and a hemagglutination assay (HA).

Adjuvant preparation

Murine CpG ODN was synthesized by Invitrogen Taiwan Ltd and given as a 10 μg per dose dissolved in the candidate vaccines. The CpG ODN sequence used was 5′-TCC ATG ACG TTC CTG ACG TT-3′ with all phosphorothioate backbones. Alum (aluminum phosphate) suspension was kindly provided from Taiwan CDC and given as a 300 μg per dose in the acidic media (pH = 6). PELC is a squalene-based water-in-oil-in-water emulsion stabilized by Span®85 (sorbitan trioleate) and PEG-b-PLACL.[10] Briefly, aqueous solution containing 120 mg of PEG-b-PLACL and 0.8 mL of phosphate buffer saline (PBS), and oil solution consisting of 0.94 mL of squalene (Sigma-Aldrich) and 0.16 mL of Span®85 (Sigma-Aldrich) were emulsified using Polytron®PT 3100 homogenizer (Kinematica AG) under 6,000 rpm for 5 min. The emulsified PELC formulation was stored at 4°C until use. PELC-formulated vaccine was investigated by re-dispersing 200 μL of stock emulsion into 1800 μL of bulk vaccine and mixed with a test-tube rotator (Labinco LD-79) under 5 rpm at least 1 h before injection. The size distribution of the formulations was determined using laser light scattering technique (Brookhaven 90 plus particle size analyzer, Brookhaven Instruments Limited).

Ethics statement and immunizations

All experiments were conducted in accordance with the guidelines of Laboratory Animal Center of NHRI. The animal use protocols have been reviewed and approved by the Institutional Animal Care and Use Committee of National Health Research Institutes (NHRI-IACUC-099073-A). Five weeks old female BALB/c mice were obtained from the National Laboratory Animal Breeding and Research Center of Taiwan and acclimatized for at least one week at the NHRI animal facility prior to use. To investigate the potency of candidate H5N1/H1N1 bivalent vaccines and/or H5N1/EV71/JEV trivalent vaccines, mice were vaccinated intramuscularly (i.m.) in quadriceps with 100 μL either once or followed prime/boost schedule. The vaccine compositions comprising 0.5 μg HA inactivated influenza viruses, 0.2 μg inactivated EV71 virus, 0.5 μg inactivated JEV virus vaccine were given either with antigen alone or formulated with Alum suspension or candidate adjuvants. Sera and spleen collection are performed to determine the B- and T-cell responses.

B-cell immunity

Serum samples were collected from immunized mice via the submandibular veins and the antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) and viral neutralizing (VN) assays, as described previously.[10,11,22] The hemagglutination inhibition (HI) activity was tested on the basis of specific anti-H5N1 antibodies to inhibit hemagglutination of turkey red blood cells (RBCs) by influenza virus HA, as it is the licensure criteria of influenza vaccines set out by the US FDA and the European Union CHMP.[11] The seroprotection was read as positive when the post-vaccination HI titer ≥ 40. Analysis of post-vaccination geometric mean titers (GMTs) is aimed at ruling out meaningful differences before and after vaccination. The statistical analysis was conducted using GraphPad Prism version 5.02 (GraphPad Software, Inc.). Data from H5N1/H1N1 viral neutralizing titers were processed by a two-tailed Student's t-test on $\log_{10}$-transformed values. Multiple comparisons of the antibody titers between groups and time points were calculated by use of the ANOVA model followed by a Bonferroni posttest on $\log_{10}$-transformed values.

T-cell immunity

To investigate H5N1-specific T-cell responses, BALB/c mice were immunized intramuscularly (i.m.) twice with 14 d between injection. Seven days after the boost, spleens were harvested and re-stimulated with 0.5 μg HA/mL of inactivated NIBRG-14 virus. Interferon (IFN)-γ and interleukin (IL)-4-secreting cells were assessed by enzyme linked immunosorbent spot (ELISPOT) assays (eBioscience) of cell suspensions for 3 d of culture, as described previously.[23]

Disclosure of Potential Conflicts of Interest

MH Huang, P Chong, CW Lin, CY Chang are named on a patent relating to multivalent vaccine against enterovirus, influenza virus, and/or Japanese encephalitis virus. This does not alter our adherence to all the policies of *Human Vaccines and Immunotherapeutics* on sharing data and materials. All other authors have no conflict of interest.

Acknowledgments

The authors are grateful to Dr Chung-Shi Yang, Center for Nanomedicine Research of NHRI for his help with the light scattering analysis. This work was supported by Department of Health of Taiwan to NIIDV of NHRI under the project of "Taiwan specific human vaccine R&D programs," and NHRI (101A1-IVPP24-014) and National Science Council of Taiwan (NSC-101-2320-B-400-011) awarded to MH Huang.

References

1. National Center for Immunization and Respiratory Diseases. General recommendations on immunization --- recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2011; 60:1-64; PMID:21293327
2. Centers for Disease Control and Prevention. Combination vaccines for childhood immunization: recommendations of the Advisory Committee on Immunization Practices (ACIP), the American Academy of Pediatrics (AAP), and the American Academy of Family Physicians (AAFP). MMWR Recomm Rep 1999; 48:1-15
3. Centers for Disease Control and Prevention. Recommended immunization schedules for persons aged 0-18 years-United States 2012. MMWR QuickGuide 2012; 61:1-4
4. American Academy of Pediatrics. Combination vaccines for childhood immunization: recommendations of the Advisory Committee on Immunization Practices (ACIP), the American Academy of Pediatrics (AAP), and the American Academy of Family Physicians (AAFP). Pediatrics 1999; 103:1064-77; PMID:10224194; http://dx.doi.org/10.1542/peds.103.5.1064
5. Spier RE. Multivalent vaccines: prospects and challenges. Folia Microbiol (Praha) 1997; 42:105-12; PMID:9306653; http://dx.doi.org/10.1007/BF02898716
6. Reddy ST, Swartz MA, Hubbell JA. Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends Immunol 2006; 27:573-9; PMID:17049307; http://dx.doi.org/10.1016/j.it.2006.10.005
7. Hubbell JA, Thomas SN, Swartz MA. Materials engineering for immunomodulation. Nature 2009; 462:449-60; PMID:19940915; http://dx.doi.org/10.1038/nature08604
8. O'Hagan DT, De Gregorio E. The path to a successful vaccine adjuvant--'the long and winding road'. Drug Discov Today 2009; 14:541-51; PMID:19508916; http://dx.doi.org/10.1016/j.drudis.2009.02.009
9. World Health Organization. WHO recommendations on pandemic (H1N1) 2009 vaccines. Geneva: Pandemic (H1N1) briefing note 2. Accessed 2009 Jul 13. Available: http://www.who.int/csr/disease/swineflu/notes/h1n1_vaccine_20090713/en/.
10. Huang MH, Huang CY, Lin SC, Chen JH, Ku CC, Chou AH, et al. Enhancement of potent antibody and T-cell responses by a single-dose, novel nanoemulsion-formulated pandemic influenza vaccine. Microbes Infect 2009; 11:654-60; PMID:19344782; http://dx.doi.org/10.1016/j.micinf.2009.03.003
11. Huang MH, Lin SC, Hsiao CH, Chao HJ, Yang HR, Liao CC, et al. Emulsified nanoparticles containing inactivated influenza virus and CpG oligodeoxynucleotides critically influences the host immune responses in mice. PLoS One 2010; 5:e12279; PMID:20808862; http://dx.doi.org/10.1371/journal.pone.0012279
12. Weeratna RD, McCluskie MJ, Xu Y, Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine 2000; 18:1755-62; PMID:10699323; http://dx.doi.org/10.1016/S0264-410X(99)00526-5
13. Liu CC, Guo MS, Lin FHY, Hsiao KN, Chang KHW, Chou AH, et al. Purification and characterization of enterovirus 71 viral particles produced from vero cells grown in a serum-free microcarrier bioreactor system. PLoS One 2011; 6:e20005; PMID:21603631; http://dx.doi.org/10.1371/journal.pone.0020005
14. Seubert A, Calabro S, Santini L, Galli B, Genovese A, Valentini S, et al. Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88. Proc Natl Acad Sci U S A 2011; 108:11169-74; PMID:21690334; http://dx.doi.org/10.1073/pnas.1107941108
15. Wilkinson TM, Li CKF, Chui CSC, Huang AKY, Perkins M, Liebner JC, et al. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. Nat Med 2012; 18:274-80; PMID:22286307; http://dx.doi.org/10.1038/nm.2612
16. Fiore AE, Uyeki TM, Broder K, Finelli L, Euler GL, Singleton JA, et al.; Centers for Disease Control and Prevention (CDC). Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. MMWR Recomm Rep 2010; 59(RR-8):1-62; PMID:20689501
17. Kelso A. CD4+ T cells limit the damage in influenza. Nat Med 2012; 18:200-2; PMID:22310682; http://dx.doi.org/10.1038/nm.2654
18. World Health Organization. Japanese encephalitis vaccines. Wkly Epidemiol Rec 2006; 81:331-40; PMID:16933380
19. Chen YJ, Hsu FK, Hsu LC. Current development and use of Japanese encephalitis vaccine. Taiwan Epidemiol Bull. 2013; 29:129-41
20. Pan SC, Kung HC, Kao TM, Wu H, Dong SX, Hu MH, et al. The Madin-Darby canine kidney cell culture derived influenza A/H5N1 vaccine: A Phase I trial in Taiwan. J Microbiol Immunol Infect 2012; S1684-1182; PMID:23022464
21. Chong P, Hsieh SY, Liu CC, Chou AH, Yuan CJ, et al. Production of EV71 vaccine candidates. Hum Vaccin Immunother 2012; 8:1-9 PMID:22992566;; http://dx.doi.org/10.4161/hv.21739
22. Chou AH, Liu CC, Chang CP, Guo MS, Hsieh SY, Yang WH, et al. Pilot scale production of highly efficacious and stable enterovirus 71 vaccine candidates. PLoS One 2012; 7:e34834; PMID:22529942; http://dx.doi.org/10.1371/journal.pone.0034834
23. Chang CY, Lin CW, Chiang CK, Chen PL, Huang CY, et al. Enzymatic stability and immunoregulatory efficacy of a synthetic indolicidin analogue with regular enantiomeric sequence. ACS Med Chem Lett. 2013; 4:522-6; http://dx.doi.org/10.1021/ml400081f